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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 3/04, 3/06, A61K 31/70 | A1

(11) International Publication Number: **WO 98/20015**

(43) International Publication Date: 14 May 1998 (14.05.98)

(21) International Application Number: PCT/GB97/03034

(22) International Filing Date: 4 November 1997 (04.11.97)

(30) Priority Data:
9622997.6 5 November 1996 (05.11.96) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

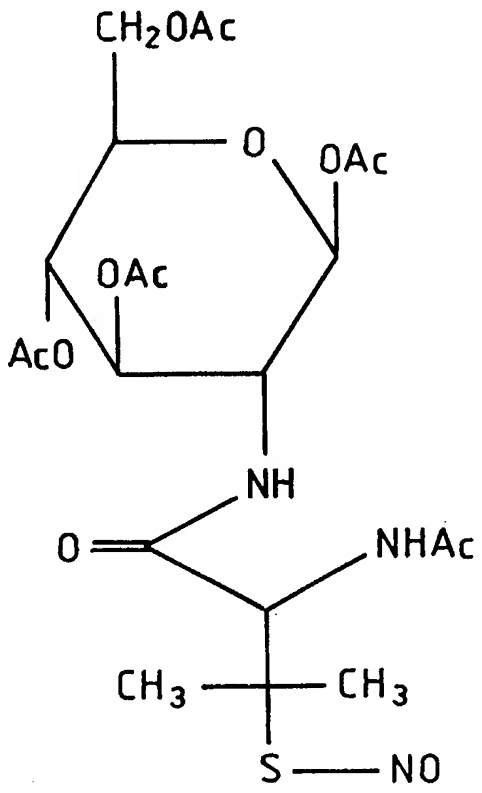
With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NITRIC OXIDE DONOR DRUGS

(57) Abstract

Disclosed is a compound capable of releasing nitric oxide from an S–nitrosothiol group (–S–N=O), the compound comprising an S–nitrosothiol group linked via an intervening moiety, to a mono–, di– or trisaccharide moiety, wherein the mono–, di– or trisaccharide moiety may be fully substituted, partially substituted, or unsubstituted, and wherein the intervening moiety serves to stabilise the S–nitrosothiol group to prevent rapid degradation thereof; together with a method of making the compound, a composition comprising the compound, and a method of causing smooth muscle relaxation.



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Title: Nitric Oxide Donor Drugs

Field of the Invention

This invention relates to novel nitric oxide donor drugs, methods of making the same, and to compositions comprising the nitric oxide donor drugs.

Background of the Invention

Nitric oxide (NO) occurs extensively in the human body. There are at least five separate physiological systems where it has been found to have an important role: as a messenger molecule in the relaxation of vascular smooth muscle; as an agent for the prevention of platelet aggregation; as a cytotoxic agent produced by the nonspecific immune system; as a messenger molecule in the brain; and as a neutrotransmitter in non-adrenergic, non-cholinergic ("NANC") nerves.

Of particular interest to the present inventors is the role of NO in the relaxation of smooth muscle (including vascular smooth muscle) and in the prevention of platelet aggregation, and the development of potential drugs to affect these and other processes.

One area of interest in the prior art is the release of nitric oxide from S-nitrosothiols (R-SNO), from the point of view their possible therapeutic use as NO-releasing drugs. S-nitrosothiols readily decompose with the release of NO and formation of a disulfide, according to equation 1:

$$(1)\ 2RSNO \rightarrow RS\text{-}SR + 2NO$$

S-nitrosothiols are very easily generated in solution from thiols by electrophilic nitrosation

(Williams 1985 Chem. Soc. Rev. *14*, 171) e.g. in aqueous solution using acidified sodium nitrite, according to equation 2:

$$(2)\ RSH + NO^+ \rightarrow RS\text{-}NO + H^+$$

Many S-nitrosothiols are too unstable in their pure form to be isolated, although it has recently been found that several RSNO compounds may be stabilised in solution by the use of Cu^+ chelating agents (Dicks *et al.*, 1996 J. Chem. Soc., Perkin Trans. *2*, 481). Some RSNO compounds, in particular SNAP (N-acetyl-S-nitrosopenicillamine) and GSNO (S-nitrosoglutathione), are stable as solids at room temperature. The structures of SNAP and GSNO are shown in Figures 1a and 1b respectively.

GSNO has been investigated for use to inhibit platelet aggregation during coronary angioplasty and also to treat a form of pre-eclampsia, a high blood pressure condition suffered by some pregnant women (Langford *et al.*, 1994 Lancet *344*, 1458; de Belder *et al.*, 1995 Lancet *345*, 124).

Another compound which has been considered as a potential drug is SNAG (1-S-Nitroso-β-D-Glucose tetraacetate), the structure of which is illustrated in Figure 1c, in which a nitrosothiol group is linked directly to a substituted glucose molecule, with no intervening moiety. Data presented by Butler *et al.* at a conference in the U.S.A. (Florida, 17-21st September 1995) and at a conference in the U.K. (St Andrew's, Scotland 25th-28th March 1996) showed that SNAG had a dilatory effect on the microvessels of the skin in human volunteers and caused dilation in the rat tail artery when applied transdermally. However, SNAG is a poor candidate for development as a therapeutic substance: it has a relatively short shelf-life which has severe practical limitations.

Of particular interest to the present inventors is a nitric oxide donor drug which may be delivered transdermally, (i.e. directly through the skin). Such a delivery route avoids the difficulties encountered with oral administration (e.g. breakdown in the stomach) and allows the drug to be delivered at a constant rate. A current example of a transdermal delivery system is the well-known nicotine patch. Transdermally delivered nitric oxide

donor drugs may be of particular use in alleviating a condition known as Raynaud's Syndrome. This afflicts as much as 5% of the population of the UK (and 80% of sufferers are female). The condition is caused by a paroxysmal or intermittent spasm of the digital arteries. The affected person suffers cold and very painful fingers and toes when exposed to lower temperatures. In more advanced cases, this can lead to cyanosis and the loss of digits through superficial gangrene.

Also of interest to the inventors is a means of treating or preventing restenosis (narrowing) and/or thrombosis of blood vessels following surgical procedures: physical damage to, or removal of, the endothelium during percutaneous transluminal angioplasty (PCTA) is a major contributory factor in the high incidence of restenosis following PCTA (Langford *et al*, Lancet **344**, 1458-1460).

Summary of the Invention

In a first aspect the invention provides a compound capable of releasing nitric oxide from an S-nitrosothiol group (-S-N=O), the compound comprising an S-nitrosothiol group linked via an intervening moiety, to a mono-, di- or trisaccharide moiety, wherein the mono-, di- or trisaccharide moiety may be fully substituted, partially substituted, or unsubstituted, and wherein the intervening moiety serves to stabilise the S-nitrosothiol group to prevent rapid degradation thereof.

The S-nitrosothiol group is conveniently linked to the intervening moiety by one or more covalent bonds and the intervening moiety conveniently similarly linked to the saccharide moiety by one or more covalent bonds.

The saccharide moiety preferably comprises a di- or monosaccharide, more preferably a monosaccharide. The saccharide units making up the mono-, di- or trisaccharide moiety are preferably pentoses or, more preferably, hexoses, although trioses, tetroses or heptoses may be employed for example.

Examples of saccharide moieties (fully or partially substituted or unsubstituted) include:

maltose, lactose, sucrose (disaccharides), ribose, arabinose, xylose (5C monosaccharides), fructose, galactose, mannose, and glucose (6C monosaccharides). Of these, the preferred saccharide is glucose.

Typically, one of the hydroxyl groups of the saccharide moiety is lost, or substituted, to allow formation of a covalent bond with the intervening moiety. Substitution of the other hydroxyl groups is not thought to have a significant effect on release of nitric oxide from the S-nitrosothiol group. However such substitution with comparatively inert groups may be preferred: those skilled in the art will appreciate that polyhydroxy compounds can be diffcult to process due to the reactivity of the hydroxyl groups (e.g. condensation reactions often occur). Substitution may be total (i.e. all the hydroxyl groups may be substituted) or may be partial (from 0-100%), but total substitution is preferred, especially where the saccharide moiety is a monosaccharide, as this avoids the difficulty of achieving selective substitution in chemical syntheses.

Conveniently the intervening moiety and/or the saccharide moiety should be substantially non-polar, as it is believed this may facilitate trans-dermal delivery and/or retention of the compound by damaged blood vessels.

Compounds of the invention are intended for use as drugs. Accordingly, whilst the substituted groups can, in theory, be any groups it is clearly preferred to avoid groups which cause toxicity, either when joined to the drug or when cleaved therefrom. Convenient substituting groups are alkyl or acyl groups (which may in turn be substituted or unsubstituted). Particularly convenient are acetyl groups, which are inert on the compound and may be cleaved from the compound *in vivo* to give safe, non-toxic acetic acid or acetate. Conveniently the substituting groups are fairly small comprising, for example, 1-4 carbon atoms, although larger substituting groups may be used if desired.

In a preferred embodiment, a hydroxyl group of the saccharide moiety is substituted by an amino group, which amino group conveniently forms an amide bond (CONH) with the intervening moiety. Accordingly preferred saccharides are amino-saccharides, especially amino monosaccharides (e.g. amino-glucose). The amino group may be at any position,

but in relation to aminoglucose position 2 is preferred, as 2-aminoglucose is a readily available compound.

As indicated above the intervening moiety, located between the saccharide moiety and the nitrosothiol group, is conveniently joined to the saccharide moiety by an amide bond. The intervening moiety is preferably of relatively small size (e.g. comprising two to twenty carbon atoms).

In a preferred embodiment, compounds according to the invention conform to the general formula shown in Figure 11, wherein R_1, R_2 and R_3 may all be the same, or may all be different or wherein any pair thereof may be the same, and wherein R_1, R_2 and R_3 may be: H, or substituted or unsubstituted alkyl, acyl or aryl groups. In particular: R_1 is preferably N-acyl (especially N-acetyl i.e. $NHCOCH_3$); R_2 is preferably alkyl (especially methyl); and R_3 is preferably alkyl (especially methyl).

The stabilising effect of the intervening moiety on the S-nitrosothiol group is due in large part to R_2 and R_3, which are substantially adjacent to the S-nitrosothiol group, and is explained in greater detail by Butler *et al.* (J. Chem. Soc. Perk. Trans *2*, in press).

Conveniently, the intervening moiety comprises penicillamine or a derivative thereof. Penicillamine is readily available commercially and has already been licensed for use as a drug (e.g. sold under the trade marks Pendramine™ and Distamine™). In particular, the intervening moiety typically comprises N-acetylpenicillamine. Figure 2 shows the structure of one compound in accordance with the invention where the intervening moiety comprises N-acetylpenicillamine.

In a second aspect, the invention provides a method of making the compound defined above, the method comprising reacting reagents under suitable conditions so as to form a compound comprising a saccharide moiety covalently linked to a nitrosothiol group via an intervening moiety. The method may also comprise the further step of isolating or substantially purifying the desired compound from other products of the reaction and/or unreacted reagents. Conventional isolation or purification techniques will be suitable for

this purpose and include, for example, fractional distillation, crystallisation, precipitation, chromatography and the like.

Compounds in accordance with the present invention have considerable utility as NO donor drugs and have surprising properties, as detailed below. In particular, the compounds are found to cause vasodilation for prolonged periods, which property was completely unexpected. Accordingly in a third aspect the invention provides a pharmaceutical composition comprising the compound defined above and a physiologically acceptable carrier. Suitable carrier substances will be well known to those skilled in the art. The invention also provides a method of making a pharmaceutical composition, comprising mixing the compound defined above with a physiologically acceptable carrier and optionally dividing the composition into unitary doses. Suitable physiologically acceptable carriers are well known to those skilled in the art and include saline or phosphate-buffered saline or inert solids (such as kaolin) and the like, the choice of which will depend, at least in part, on the proposed route of delivery of the composition.

Compounds in accordance with the invention may be delivered to a subject by injection (e.g. sub-cutaneous, intra-muscular, intra-venous routes). In one embodiment, especially useful in the treatment of Reynaud's Syndrome, the compounds are delivered trans-dermally. Thus in a fourth aspect the invention provides a skin covering for application to the skin of a mammalian (typically human) subject, the skin covering comprising an effective amount of a compound in accordance with the first aspect of the invention. What constitutes an effective amount of the compound will vary slightly with the activity of the particular compound in question, but those skilled in the art will readily be able to determine an appropriate amount by routine trial and error. Typically an effective amount will vary between 1mg and 10 grams of the compound (more normally between 10mgs and 1 gram).

The skin covering conveniently comprises an adhesive for releasable attachment to the skin of a human subject, such as the adhesives used in self-adhesive nicotine patches or sticking plasters. Preferably the skin covering takes the form of a patch, plaster, bandage, gauze, wound dressing or the like, impregnated, coated or otherwise treated with a compound in

accordance with the first aspect of the invention.

As described previously, NO donor drugs may exert a number of effects on a human subject, including relaxation of smooth muscle (especially vascular smooth muscle, leading to vaso-dilation). Thus in a fifth aspect, the invention provides a method of causing relaxation of smooth muscle (especially including vascular smooth muscle, leading to vaso-dilation) of a mammalian (desirably human) subject, the method comprising delivering to the subject an effective amount of a compound in accordance with the first aspect of the invention. The compound is preferably delivered transdermally (for example, by use of a patch, plaster, bandage, gauze, wound dressing or the like defined above). The method of causing vasodilation may be especially useful in promoting wound healing and/or in the treatment of Raynaud's Syndrome.

In addition, the compounds of the invention may be used to prevent or treat narrowing of blood vessels whose endothelial integrity has been impaired (e.g. due to performance of surgical procedures such as percutaneous transluminal angioplasty ["PCTA"] or coronary artery-bypass grafting ["CABG"]). Thus, for example, following PCTA blood vessels may be treated with a compound in accordance with the invention as a preventative or therapeutic measure.

In a further aspect, the invention provides for use of the compound defined above in the preparation of a medicament to cause relaxation of smooth muscle in a mammalian (typically human) subject. Typically the medicament is used for the relaxation of vascular smooth muscle, such that use of the medicament leads to vasodilation in a subject. The medicament may be used to treat a number of conditions, such as Raynaud's Syndrome, or to prevent restenosis of blood vessels following surgical procedures (e.g. PCTA or CABG), as described above, but other conditions which are ameliorated by smooth muscle relaxation may also be usefully treated by the medicament.

The invention will now be described with reference to the following illustrative examples and to the drawings, in which:

Brief Description of the Figures

Figures 1a, 1b and 1c illustrate the structures of prior art S-nitrosothiol compounds;

Figure 2 shows the structure of a compound in accordance with the invention, having use as a nitric oxide donor drug;

Figure 3 is a schematic illustration of a route by which the compound shown in Figure 2 may be synthesised;

Figures 4a-4d are pressure recordings showing vasodilator responses to sequential bolus micro-injections (10 μl) of SNAP (4a and 4b) or RIG200 (4c and 4d; log M concentrations as indicated) into the perfusate of endothelium-intact and endothelium-denuded vessels. Perfusion with Hb (10 μM) is indicated by the solid bar. The bar at the lower left of the figures indicates scale (vertically, a 25% response; horizontally, 5 minutes).

Figures 5a-d show Log-dose response curves (% pressure change against $\log_{10}$ M bolus concentration) demonstrating the vasodilator effect of bolus micro-injections (10 μl) of SNAP (5a and 5b) or RIG200 (5c and 5d) on endothelium-intact (5a and 5c) and -denuded (5b and 5d) rat femoral arteries. Peak amplitude (filled triangles) and recovery (open triangles) of responses are illustrated (all n = 8). Points shown are means and vertical lines indicate s.e. mean.

Figures 6a and 6b show Log-dose response curves showing the vasodilator effect (% pressure change) of bolus micro-injections (10 μl) of SNAP (6a) or RIG200 (6b) in rat endothelium-denuded femoral arteries whilst being perfused with 10 μM Hb. Peak amplitude (filled symbols) and recovery (open symbols) of responses is illustrated (all n = 8). Points show means and vertical lines indicate s.e. mean.

Figures 7a-c show pressure recordings of vasodilator responses to single bolus micro-injections of 10^{-3} M RIG200 in: (7a) an endothelium-intact vessel in the absence of L-NAME; and (7b) an endothelium-denuded vessel in the absence of L-NAME but perfused

with Hb (10 μM) for the period indicated; and (7c) an endothelium-denuded vessel perfused with L-NAME for the period indicated (3 h). The scale marker on the left indicates (vertically) a 25% response and (horizontally) 30 minutes.

Figures 8-10 illustrate schematically the synthesis of certain compounds in accordance with the invention; and

Figure 11 shows the general formula of compounds according to a preferred embodiment of the invention.

Example 1

One compound in accordance with the present invention has the structure shown in Figure 2, and for convenience has the code name RIG200. The inventors have synthesised and authenticated the compound and conducted biological assays. RIG200 has the formal chemical name *N*-(*N*-acetyl-*S*-nitrosopenicillaminyl)-2-aminoglucose tetraacetate.

RIG200 has been synthesised in the laboratory, via the route illustrated schematically in Figure 3. The steps shown in Figure 3 involve the following reactions:

(i) Anisaldehyde/NaOH

(ii) Acetic anhydride/pyridine

(iii) HCl

(iv) Sodium acetate

(v) *N*-acetylpenicillamine/1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate/dichloromethane

(vi) Sodium nitrite in acid

The synthesis is described in more detail below:

Anisol glucosamine was prepared as follows: Glucosamine hydrochloride (20 g) was dissolved in 1 M NaOH (94 ml). Anisaldehyde was added (11.4 ml) and the mixture shaken until a white precipitate was seen. The flask was cooled in a freezer for 10 mins

and filtered. The white solid was washed with ice water and chilled ether. The solid was dried in a vac oven to give a white product (26.2 g, 95.1%). m.p. 163°C (lit. 166°C). δ_H (200 MHz, DMSO) 2.85 (1 H, t, J = 8.5 Hz, 2-H), 3.2 (1 H, m, 5-H), 3.85 (3 H, s, OMe), 4.65 (1 H, t, J = 5.6 Hz), 4.75 (1 H, t, J = 7.2 Hz,), 4.9 (1 H, d, J = 4.8 Hz,), 6.6 (1 H, d, J = 6.6 Hz, 1-H), 7.0 (2 H, d, J = 8.6 Hz, aromatics), 7.7 (2 H, d, J = 8.6 Hz, aromatics), 8.15 (1 H, s, CH = N). δ_C(200 MHz, DMSO) 55.54 (OMe), 61.52 (C2), 70.61, 74.80, 77.07 and 78.40 (C3 - C6), 95.85 (C1), 114.18 and 129.94 (aromatics), 161.71 (CHN).

Anisol glucosamine tetra-O-acetate was prepared as follows: The above product (15 g) was cooled with stirring in a mixture of pyridine (81 ml) and acetic anhydride (45 ml). The mixture was left to stand at room temperature overnight and then poured into ice water with stirring. A white precipitate appeared and was filtered, dried and recrystallised from ethanol. m.p. 187°C (lit.[16] 188°C). δ_H (200 MHz, $CDCl_3$) 1.85 - 2.05 (12 H, m, OAc), 3.4 (1 H, t, J = 8.6 Hz, 2-H), 3.8 (3 H, s, OMe), 3.95 (1 H, m, 5-H), 4.05 (1H, dd, J = 12.6, 2.0 Hz, 6 or 6'-H), 4.35 (1 H, dd, J = 12.4, 4.6 Hz, 6 or 6'-H), 5.1 (1 H, t, J = 9.6 Hz, 3 or 4-H), 5.4 (1 H, t, J = 9.4 Hz, 3 or 4-H), 5.95 (1 H, d, J = 8.2 Hz, 1-H), 6.9 (2 H, d, J = 8.6 Hz, aromatics), 7.65 (2 H, d, J = 8.6 Hz, aromatics) and 8.15 (1 H, s, CHN). δ_C (200 MHz, $CDCl_3$) 20.96, 21.1 and 21.24 (OAc), 55.87 (OMe), 62.27 (C2), 68.49, 73.23, 73.37 and 73.70 (C3 - C6), 93.62 (Cl), 114.54 and 130.74 (aromatics) and 164.77 (CHN).

Glucosamine tetra-O-acetate hydrochloride was prepared as follows: The product from above was dissolved in the minimum amount of acetone with heating. A small quantity of water was added and the mixture cooled. Concentrated HCl was added dropwise until a precipitate was seen. Chilling was continued whilst stirring with ether, to remove the anisaldehyde. The solid was filtered and washed with chilled ether and was found not to melt (as in the literature). δ_H (300 MHz, D_2O) 2.0 (12 H, m, OAc), 3.6 (1 H, t, J = 10 Hz, 2-H), 4.1 (3 H, m, 5, 6 and 6'-H), 5.0 (1 H, t, J = 10 Hz, 3 or 4-H), 5.4 (1 H, t, J = 10 Hz, 3 or 4-H) and 5.9 (1 H, d, J = 10 Hz, 1-H). δ_C (200 MHz, D_2O) 22.93 and 23.05 (OAc), 55.14, 64.27, 70.83, 73.60, 74.98 (C2 - C6) and 93.16 (Cl).

Glucosamine tetra-O-acetate was prepared as follows: the above product (4.7 g) was dissolved in water. NaOAc (3.4 g, 2 eq) was added and a white suspension formed. The suspension was extracted with DCM (3 x 50 ml), dried ($MgSO_4$) and evaporated. Recrystallisation from ether gave a white solid. δ_C ($CDCl_3$) 21.07, 21.21 and 21.40 (OAc), 55.50, 62.21, 68.68, 73.13 and 75.50 (C2 - C6) and 95.65 (Cl) mpt 142°C (lit 143°C) 1H nmr 5.45 (1H, d, J = 8.6Hz, 1-H).

Glycopeptide "GLUPEN" was prepared as follows: Glucosamine acetate (1.3 g) and N-Acetyl penicillamine (0.7 g) were stirred in DCM. The penicillamine remains as a suspension. The coupling agent 1-Cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluene sulphonate (1.6 g, 1 eq.) was added and the mixture stirred at room temperature. The suspension briefly went into solution before a white precipitate appeared. Stirring was continued for 24 hrs. The solution was washed with 1 M HCl, saturated $KHCO_3$, and water, then dried ($MgSO_4$) and evaporated. Recrystallisation from ether gave a pungent white solid. δ_C (200 MHz, $CDCl_3$) 21.09, 21.19, 21.37 (OAc), 23.48 (NAc), 28.91 and 31.25 ($HSC(\underline{C}H_3)$, 45.68 ($HS\underline{C}(CH_3)$, 52.75, 60.63, 62.224, 68.68+69.12, 71.98, 72.97+73.23, 92.07+92.77 (Cl) and 170 - 171 (quaternaries). Found: C, 48.32, H, 6.10, N, 5.25%, $C_{21}H_{32}N_2O_{11}S$ required: C, 48.46, H, 6.15, N, 5.38%.

S-nitroso "GLUPEN" (RIG 200) was prepared by the nitrosation of the above product with $HCl/NaNO_2$ and extraction into DCM. Evaporation and addition of ether gave a stable green solid with λ_{max} at 340 nm. δ_C (200 MHz, $CDCl_3$) 20.89 and 21.18 (OAc), 23.63 (NAc), 26.21 and 26.57 ($ONSC(\underline{C}H_3)$, 53.93, 57.91 ($ONS\underline{C}(CH_3)$, 60.65, 62.21, 68.57+68.94, 73.08+73.15, 92.01 (Cl) and 170 - 171 (quaternaries).

(The literature ("lit.") referred to above is: Bergmann & Zervas 1931 Chem. Ber. *64*, 975-979).

Example 2 Characterisation of RIG200

The precursors (shown in Figure 3) of RIG200 are all known compounds. The immediately preceding thiol compound has been characterised by ^{13}C NMR spectroscopy

and by a satisfactory elemental analysis. The S-nitrosothiol (i.e. RIG200) itself has been characterised by the inventors using ^{13}C NMR spectroscopy, by the characteristic visible spectrum of an SNO group, and by mass spectroscopy.

Biological Assays

Experiments were carried out to determine the biological activity of RIG200 in terms of its ability to cause vasodilation in lengths of rat femoral artery. Responses to RIG200 were compared with those obtained with the known compound *S*-nitroso-*N*-acetylpenicillamine (SNAP) and were inhibited with ferrohaemoglobin to establish whether NO was involved in the observed vasodilation. Experiments were performed on both endothelium-intact vessels, and on vessels where the endothelium was denuded by passage of air through the lumen.

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (400-550 g; $n = 36$). The perfusion system used was similar to that described previously for rat tail artery perfusion (Flitney *et al*, Br. J. Pharmacol. **107**, 842-848).

Methods

Briefly, animals were killed by cervical dislocation and both femoral arteries were exposed and cannulated immediately distal to the epigastric arterial branch. Cannulated arterial segments (7-8 mm long) were dissected free and transferred to perspex organ bath chambers (1 ml volume) at 37°C where they were perfused (0.6 ml min^{-1}; Gilson minipuls 3, Anachem, Luton, UK) and superfused (1 ml min^{-1}, Watson Marlow 3025; Watson Marlow, Falmouth, UK) with fresh oxygenated Krebs buffer solution. Twin vessels were precontracted with phenylephrine (PE) and perfusion pressure was monitored by a differential pressure transducer (T; Sensym SCX 15ANC, Farnell Electronic Components, Leeds, UK) located upstream.

Analysis of results

Signals from the pressure transducers were processed by a MacLab/4e analogue-digital

converter and displayed through 'Chart' software (AD Instruments, Sussex, UK) on a Macintosh Performa 630 microcomputer. Vasodilator response amplitude was expressed as a % of PE-induced pressure existing before the first in a series of drug applications (% pressure change; negative values represent relaxation, positive represent constriction). Data are given as % pressure change both at the peak of responses and following response recovery as defined earlier. Mean values are given $\pm$s.e. mean.

P values stated in the text were obtained by two-factor, repeated dose ANOVAs except where stated otherwise. Unpaired, two-tail student's t-tests and Mann-Whitney U (MWU) non-parametic tests were also used where appropriate. $P<0.05$ was accepted as statistically significant.

The apparatus permits exclusive drug delivery to the luminal surface of the vessel by bolus injection (10 μl) through a resealable rubber septum into the perfusate immediately upstream of the vessel (transit time to artery ~ 3 s, through lumen ~ 300 ms). Injections of vehicle (Krebs buffer) had no effect on perfusion pressure. Vasodilator responses in control vessels could be compared to those perfused with supramaximal concentrations of either the recognised NO scavenger, ferrohaemoglobin (Martin *et al*, 1985 J. Pharmacol. Exp. Ther. **232**, 708-716; Hb; 10 μM), or the NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester, abbreviated as L-NAME (Rees *et al*; 1990 Br. J. Pharmacol. **101**, 746-752), at 200 μM. Where possible, two vessels from each animal were used in parallel; one receiving SNAP and the other RIG200.

Experimental protocols. All experiments were carried out in a darkened laboratory in order to protect photolabile drugs and to prevent photorelaxation of vessels (Megson *et al*, 1995 Endothelium **3**, 39-46). All drugs were dissolved and diluted in PE-containing Krebs solution and kept on ice before use.

Once precontracted, endothelial function was assessed by use of the endothelium-dependent vasodilator, carbachol (CCh). Bolus injections of supra-maximal concentrations of CCh (10 μl; 10mM) into the perfusate of endothelium-intact vesels caused transient vasodilatations similar to those described in rat tail arteries (Flitney *et al*, 1992 cited

above). In experiments where the endothelium was removed, air was passed through the lumen until such time as the vessel was unresponsive to CCh (5-10 min).

Removal of the endothelium invariably caused an increase in pressure due to loss of endothelium-derived NO synthesis. Pressure was restored to its original level by appropriate reduction in PE concentration (~0.5 x original concentration). Selected vessels were taken for histological staining to confirm endothelial removal (5 μm paraffin sections, fixed in formalin (10%; 24 h) and stained with haematoxylin and eosin). Final assessment of the integrity of the endothelium was achieved at the end of experiments where vessels were perfused with ferrohaemoglobin (Hb; 10 μM). Perfusion pressure in endothelium-intact vessels rose considerably higher than pretreatment pressure, indicating the presence of endothelium-derived NO, whilst endothelium-denuded vessels failed to develop pressures above pretreatment levels.

Vasodilator responses to bolus injections of SNAP and RIG200. Bolus injections of increasing concentrations of SNAP or RIG200 (10 μl; 10^{-8}-10^{-3}M) were made sequentially into the perfusate of precontracted, endothelium-intact or denuded vessels. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next concentration was injected. Time intervals between injections of SNAP and RIG200 were matched for each individual experiment. Responses to 10^{-3}M concentrations were then allowed to recover for periods of between 15 min and 4 h, after which vessels were perfused with Hb (10 μM).

The effect of Hb on vasodilator responses to SNAP and RIG200. In order to assess the role of NO in SNAP and RIG200-induced vasodilatation, sequential bolus injection experiments were also carried out in endothelium-denuded vessels perfused with Hb (10 μM).

The effect of L-NAME on sustained vasodilator responses to RIG200. The possible role of the inducible isoform of NOS (iNOS) in sustaining RIG200-induced vasidilatation in endothelium-denuded vessels was assessed by examining the effect of perfusing vessels with the NOS inhibitor, L-NAME (200 μM; 3h), on responses to a single bolus injection

(10^{-3}M) of RIG200 in endothelium-denuded vessels. Perfusion commenced 1 h after the bolus injection of RIG200.

Results

Biological activity of SNAP and RIG200

Vessel parameters. Vessels were precontracted with PE (10.2±0.3 μM) in order to give pressures of ~90 mm Hg (91.8±4.0 mmHg; n=52). Endothelium intact vessels dilated in response to 10 mM bolus injections of CCh (25.5 ± 3.3% relaxation; n=22). Pressure in PE-contracted vessels increased by 52.3±9.3% (n=14) when perfused with 10 μM Hb, confirming the presence of a functional endothelium.

Endothelium removal, by perfusion of air, abolished responses to CCh and caused an increase in PE-induced pressure of 75.4±11.3% (n=30). Pressure in these vessels failed to rise significantly above baseline pressure when perfused with Hb (+4.5±4.8%; n=14).

Examination of histological sections of vessels confirmed the presence of endothelium in intact vessels and successful removal in those perfused with air.

Vasodilator responses to bolus injections of SNAP and RIG200. Bolus micro-injections of SNAP into the perfusate of endothelium-intact vessels caused concentration-dependent, transient vasodilations (Figures 4a and 5a). Responses to moderate concentrations of SNAP typically recovered to pressures higher than pre-injection pressure. The effect subsided, but pressure often remained slightly elevated (+11.2±8.1% for 10^{-5}M SNAP; P=0.042). Responses to the highest concentration of SNAP (10^{-3M}M) caused relaxations of -75.2±4.7% and generally recovered fully in <20 min. However, some vessels (n=2/7) failed to recover to pre-injection pressures even after washout periods of up to 1 h. Perfusion with 10 μM Hb invariably caused pressure to rise significantly above pre-injection pressures (+45.1± 15.1%; P=0.001).

Endothelium removal did not significantly increase vessel sensitivity to SNAP (P=0.75). However, the contractile effect often seen in endothelium-intact vesels following SNAP-induced vasodilatations was not seen in denuded vessels (Figures 4b and 5b). Responses to the highest concentration of SNAP (10^{-3}M) in some vessels (n=2/7) failed to recover

to pre-injection pressures even after washout periods of up to 1 h. Perfusion with 10 μM Hb following this concentration of SNAP caused rapid recovery to pre-injection pressures (+7.9 $\pm$ 3.8%) in those vessels which had failed to show full spontaneous recovery (Figure 4b).

Responses to RIG200 in endothelium-intact vessels were transient and similar in nature to those for SNAP (Figures 4c and 5c). Perfusion of these vessels with Hb invariably caused a rapid rise in pressure to +74.1$\pm$5.4% (P=0.001; two-tailed, unpaired Student's t rest) above pre-injection levels. Responses in endothelium-denuded vessels also showed full recovery following 10^{-3}-10^{-7}M microinjections but thereafter failed to recover to pre-injection pressure (Figures 4d and 5d). The sustained vasodilator effect of moderate and high concentrations of RIG200 was concentration-dependent, with perfusion pressure recovering to only 48.0$\pm$5.2% of pre-injection pressure following a 10^{-3}M bolus injection. The response persisted for >4h and was fully reversed on perfusion with Hb, reaching pressures +1.0$\pm$3.2% of pre-injection levels. The rate of pressure recovery on perfusion with Hb was often noticeably slower in vessels treated with RIG200 than SNAP.

Vessels were less sensitive to RIG200 than SNAP in either endothelium-intact (P=0.022) or endothelium-denuded vessels (P=0.014), corresponding to greater stability of RIG200 *in vitro*. Relaxations in response to 10^{-3}M RIG200 were similar in amplitude to those to SNAP (75.0$\pm$3.0% relaxation). Endothelium-removal did not significantly alter sensitivity to RIG200 (P=0.66).

Delivery of maximal and supra-maximal concentrations of both SNAP and RIG200 was limited by their saturation points in Krebs buffer (saturated at $\sim$3 x 10^{-3}M), preventing calculation of EC_{50} values.

The effect of Hb on vasodilator responses to SNAP and RIG200 in denuded vessels. The peak amplitude of responses to bolus injections of SNAP in endothelium-denuted vessels was significantly attenuated, but not abolished, in the presence of Hb (P=0.001; Figure 6a). Similarly, perfusion of endothelium-denuded vessels with Hb caused inhibition of RIG200-induced responses (P=0.019) and abolished the sustained vasodilator effect of

RIG200 (Figure 6b).

The effect of L-NAME on sustained vasodilator responses to RIG200. Single bolus micro-injections of RIG200 (10^{-3}M) in endothelium-intact vessels caused transient vasodilatations which recovered to 95.3±4.2% in <30 min (Figure 7a; n=8). Equivalent micro-injections of RIG200 in endothelium-denuded vessels caused relaxations which only recovered to 48.2±7.9% of pre-injection pressure 4 h after injection (Figure 7b; n=6). Sustained vasodilatation was inhibited by Hb, as described previously, and the effect was reversed on washout (Figure 7b; n=6). However, sustained vasodilatation in endothelium-denuded vessels was not significantly affected by L-NAME (Figure 7c), with perfusion pressure recovering to 45.3 ± 3.2% of pre-injection pressure 4 h after bolus washout (n=6; P=0.47 compared to untreated vessels; unpaired Student's t test).

Experiments were also conducted to investigate the decomposition of RIG200 *in vitro*, in comparison with that of SNAP, in oxygenated Kreb's solution at 24°C (data not shown).

The inventors have found that RIG200 decomposes spontaneously in solution to release NO *in vitro* and is capable of causing NO-mediated vasodilatation in rat isolated femoral arteries. Decomposition was found to be slower than that of the parent compound, SNAP, but was not susceptible to trace Cu(I) catalysis *in vitro*. Bolus injections of both SNAP and RIG200 produced transient responses in endothelium-intact, rat isolated femoral arteries. SNAP-induced vasodilatations in endothelium-denuded vessels were transient whilst those induced by RIG200 were sustained for up to 4 h.

Spectrophotometric analysis showed RIG200 to be 5 fold more stable than SNAP in Krebs buffer solution. The rate of SNAP decomposition *in vitro* is notoriously variable, a feature which is now understood to be due to differences in the amounts of trace Cu(I) present in solutions (Dicks *et al*, 1996). It has been suggested that a small proportion of Cu(II) is reduced to Cu(I) by thiols present as impurities in solutions of S-nitrosothiols and that it is Cu(I) which catalyzes S-nitrosothiol decomposition (Dicks *et al*, 1996).

Decomposition of both SNAP and RIG200 would be expected to be greater *in vivo* since

Cu(II) levels in human serum are 12-24 μM of which 1-2 μM is unbound (Lenter, 1984 Geigy Scientific Tables, p86, Basel, Switzerland, Ciba-Geigy Ltd). The relatively slow, trace Cu(I)-resistant decomposition of RIG200 *in vitro* is advantageous in limiting the undesirable variability in NO generation and the resultant handling difficulties experienced with SNAP, and may influence its biological activity.

Bolus injections of SNAP or RIG200 into the perfusate of precontracted, endothelium-intact rat isolated femoral arteries induced transient vasodilatations (Figures 4 and 5). Vessels were significantly less sensitive to RIG200 than to SNAP, perhaps reflecting the relative stability of RIG200 in solution. NO is known to inhibit synthesis of endogenous NO by endothelial NOS and this process may be responsible for the modest vasconstriction seen following responses to bolus injections of intermediate concentrations (10^{-5}-10^{-4}M) of either SNAP or RIG200 in endothelium-intact vessels (Figures 4a,c and 5a,c). Absence of equivalent vasoconstriction in endothelium-denuded vessels (Figures 4b,d and 5b,d) is further evidence for NO-induced inhibition of NOS by NO derived from S-nitrosothiols.

Endothelium-removal did not significantly affect vasodilations induced by bolus injections of SNAP or RIG200 (Figures 4a,c and 5a,c) and responses to both compounds were significantly inhibited by Hb (Figure 6a,b). However, responses to intermediate and high concentrations of RIG200 in endothelium-denuded vessels recovered at a slower rate (Figure 4d) and caused a sustained depression of tone (Figures 4d and 5d). Pre-injection pressure was restored by perfusing with Hb, confirming that sustained vasodilatation is entirely NO-mediated. The slow rate of recovery observed in RIG200-treated vessels (Figure 4d) may indicate that NO generated by RIG200 is less accessible to Hb than that derived either from the endothelium or from SNAP. The inhibitory effect of Hb on sustained vasodilatation was reversible on washout (Figure 7b), indicating continuing NO generation after Hb treatment.

Vasodilatation to a single bolus injection of 10^{-3}M RIG200 in endothelium-intact vessels typically recovered to pre-injection pressure within 30 min (Figure 7a). Equivalent responses in endothelium-denuded vessels caused sustained vasodilatation which showed only partial recovery after 4 h washout (Figure 7b). Sustained responses were unaffected

by L-NAME treatment (Figure 7c), excluding induction of iNOS as a possible mechanism for the effect.

A possible explanation for these results is that SNAP-induced, transient vasodilatations are caused primarily by spontaneous release of NO in the perfusate, irrespective of the presence of endothelium. Similarly, transient responses to RIG200 in endothelium-intact vessels are due to spontaneous release of NO in the perfusate. However, responses to RIG200 in endothelium-denuded vessels appeared to comprise two elements; a transient component due to spontaneous NO release in the perfusate and a sustained component due to release of NO from RIG200, retained within the tissue long after the bolus has passed through the lumen. It is apparent that the sustained component of vasodilator responses to RIG200 is specific to endothelium-denuded vessels. We suggest that endothelial cells may present a barrier to RIG200 retention but that in endothelium-denuded vessels, RIG200 may be retained in the tissue underlying the endothelium (the internal elastic lamina and/or the media). Possible retention of RIG200 by the sub-endothelium may be particularly important in view of emerging evidence that the barrier function of endothelium is altered by oxidized low density lipoprotein (Rangaswamy *et al*, 1997 Circ. Res. **80**, 37-44), which is known to accumulate in atherosclerotic lesions (Haberland *et al*, 1988 Science **241**, 215-218).

The stability of RIG200 *in vitro* is a distinct advantage over existing S-nitrosothiols with regard to its handling, and may be a factor in increasing the duration of vasodilator responses *in vivo*. In addition, the ability of RIG200 to cause selectively sustained vasodilatation in vessels where the endothelium has been removed could have important implications in cardiovascular diseases, where endothelial damage is a contributory factor, and may be useful in preventing restenosis of vessels following surgical procedures like PCTA and CABG (coronary artery-bypass grafting).

Having established the potential of RIG200 as a vasodilator drug, the inventors have devised synthetic routes for analogous compounds from aminoglucoses where the amino group is at positions 1, 3 and 6. These analogues may be equally useful as therapeutic compounds, being structurally similar to RIG200. The synthetic routes are described in

the following examples.

Example 3

Synthesis of *N*-(*N*-acetyl-*S*-nitrosopenicillaminyl)-1-aminoglucose tetraacetate.

The synthesis is illustrated schematically in Figure 8. Steps (i)- (iv) involve the following reactions:

(i) Sodium azide/DMF
(ii) Pd/C and hydrogen in methanol
(iii)N-acetylpenicillamine/1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate/dichloromethane
(iv) Sodium nitrite in acid

Example 4

Synthesis of *N*-(*N*-acetyl-*S*-nitrosopenicillaminyl)-3-aminoglucose tetraacetate.

The synthesis is illustrated schematically in Figure 9. Steps (i)-(vii) involve the following reactions.

(i) acetone/zinc chloride/phosphoric acid
(ii) Trifluoromethanesulphonic anhydride/pyridine/dichloromethane
(iii) 1. Tosic acid/acetonitrile 2. acetic anhydride/pyridine
(iv) Sodium azide/DMF
(v) Pd/C and hydrogen in methanol
(vi) N-acetylpenicillamine/1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate/dichloromethane
(vii) Sodium nitrite in acid

Example 5

Synthesis of *N*-(*N*-acetyl-*S*-nitrosopenicillaminyl)-6-aminoglucose tetraacetate.

The synthesis is illustrated schematically in Figure 10.

Steps (i)-(iv) involve the following reactions:

(i) Tosyl chloride/pyridine

(ii) Sodium azide/DMF

(iii) Pd/C and hydrogen in methanol

(iv) N-acetylpenicillamine/1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate/dichloromethane

(v) Sodium nitrite in acid

CLAIMS

1. A compound capable of releasing nitric oxide from an S-nitrosothiol group (-S-N=O), the compound comprising an S-nitrosothiol group linked via an intervening moiety, to a mono-, di- or trisaccharide moiety, wherein the mono-, di- or trisaccharide moiety may be fully substituted, partially substituted, or unsubstituted, and wherein the intervening moiety serves to stabilise the S-nitrosothiol group to prevent rapid degradation thereof.

2. A compound according to claim 1, wherein the saccharide moiety comprises a monosaccharide.

3. A compound according to claim 1 or 2, wherein the intervening moiety and/or the saccharide moiety are substantially non-polar.

4. A compound according to any one of claims 1, 2 or 3, wherein the saccharide moiety is joined to the intervening moiety by an amide bond.

5. A compound according to any one of the preceding claims, according to the general formula shown in Figure 11, wherein R_1, R_2 and R_3 may be any one of: H, or substituted or unsubstituted alkyl, acyl or aryl groups.

6. A compound according to claim 5, wherein R_1 is N-acyl, and R_2 and R_3 are alkyl.

7. A compound according to any one of the preceding claims, having the structure shown in Figure 2.

8. A method of making a compound according to any one of claims 1-7, comprising reacting reagents under suitable conditions so as to form a compound comprising a saccharide moiety linked to a nitrosothiol group via an intervening moiety.

9. A method according to claim 8, further comprising the step of isolating or substantially purifying the compound from other products of the reaction and/or unreacted reagents.

10. A pharmaceutical composition comprising a compound in accordance with any one of claims 1-7, together with a physiologically acceptable carrier.

11. A method of making a pharmaceutical composition, comprising mixing a compound in accordance with any one of claims 1-7 with a physiologically acceptable carrier and, optionally, dividing the composition into unitary doses.

12. A skin covering for application to the skin of a mammalian subject, the skin covering comprising an effective amount of a compound in accordance with any one of claims 1-7.

13. A skin covering according to claim 12, comprising an adhesive for releasable attachment of the covering to the skin of a human subject.

14. Use of a compound in accordance with any one of claims 1-7 in the preparation of a medicament to cause smooth muscle relaxation in a mammalian subject.

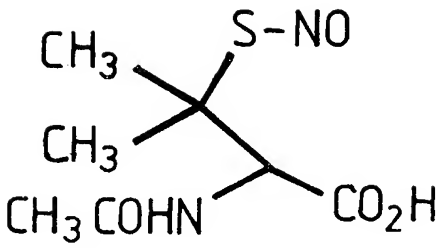
15. A method of causing smooth muscle relaxation in a mammalian subject, the method comprising administering to the subject an effective amount of a compound in accordance with any one of claims 1-7.

16. A method according to claim 15, which causes vasodilation in the subject.

17. A method according to claim 15 or 16, wherein the compound is administered by injection or transdermally.

18. A method according to any one of claims 15, 16 or 17, for treatment of Raynaud's Syndrome or to prevent restenosis of blood vessels following surgical procedures.

19. A compound substantially as hereinbefore described and with reference to the accompanying drawings.



SNAP

Fig. 1a

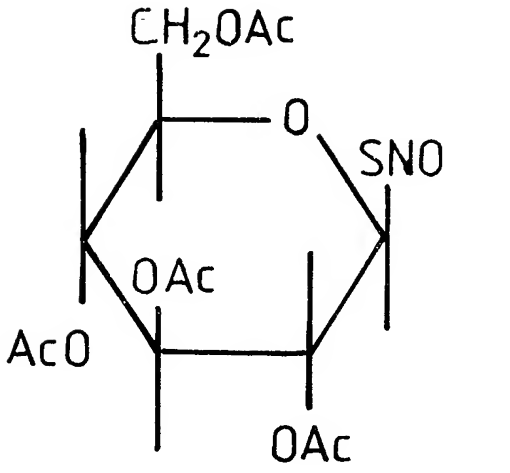
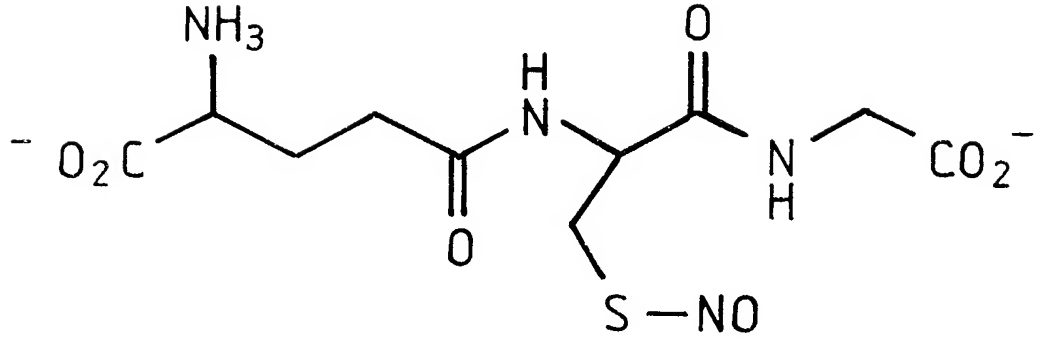


Fig. 1c



GSNO

Fig. 1b

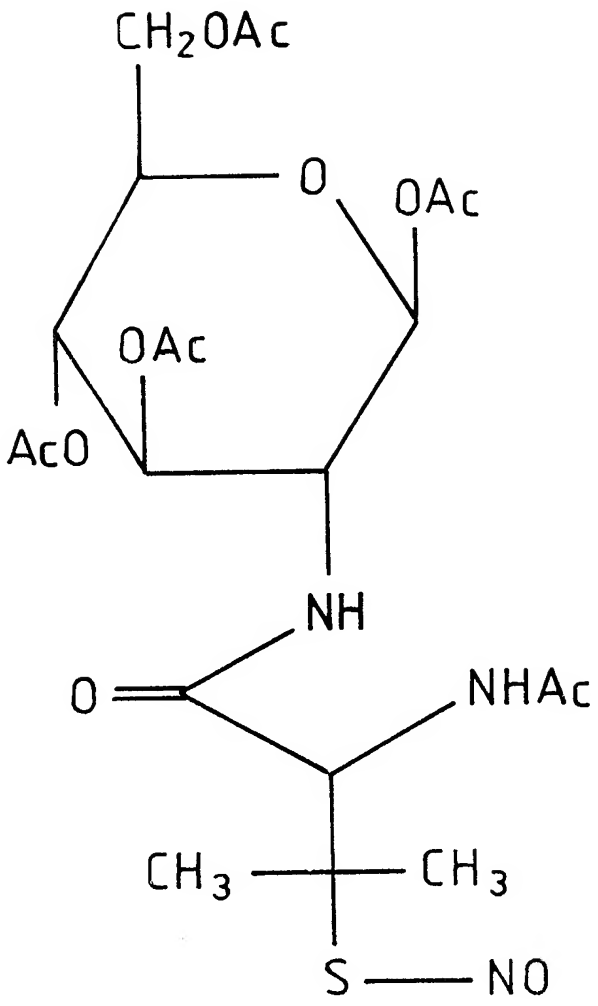
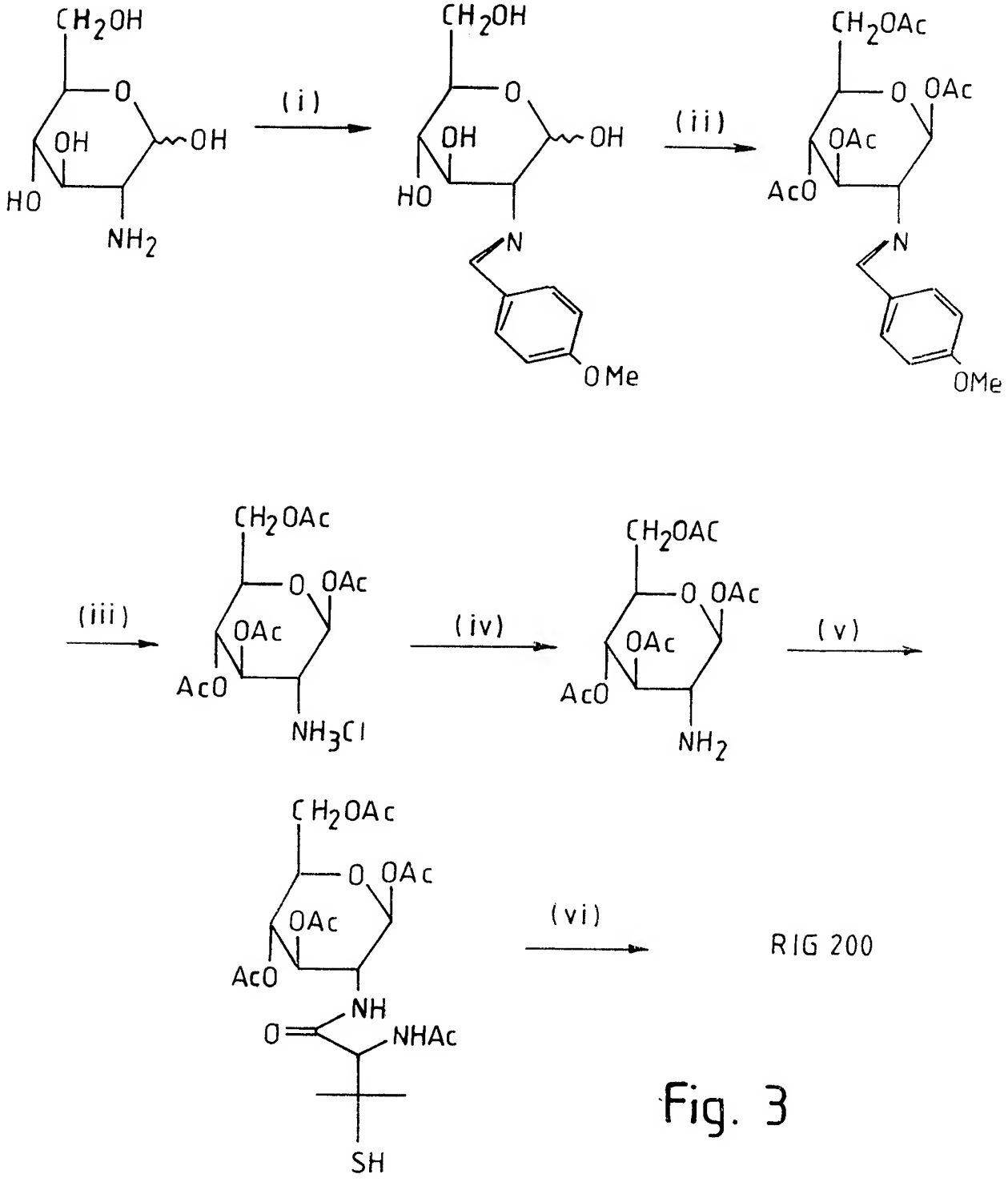


Fig. 2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RIG 200

Fig. 3

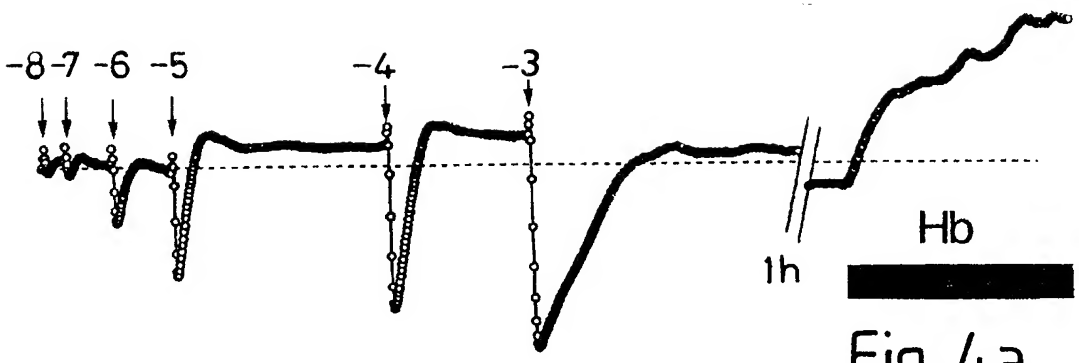

Fig. 4a

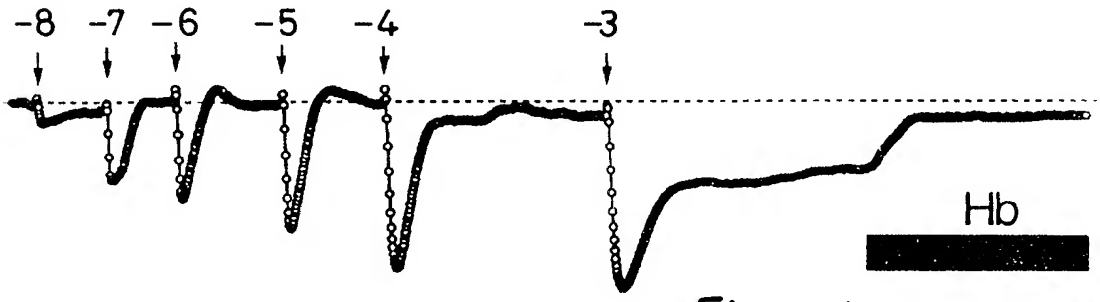

Fig. 4b

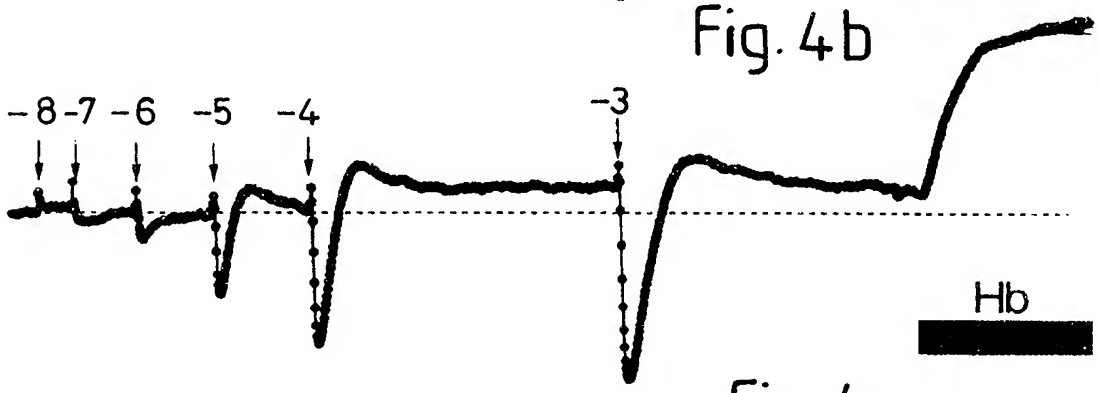

Fig. 4c

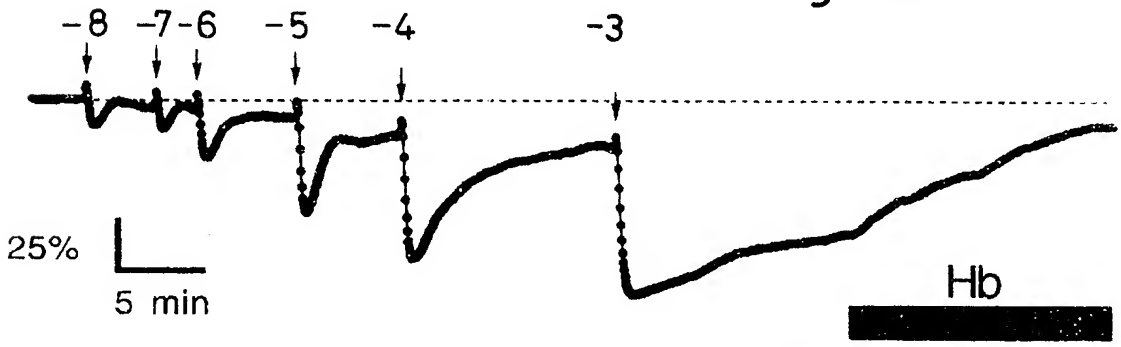

Fig. 4d

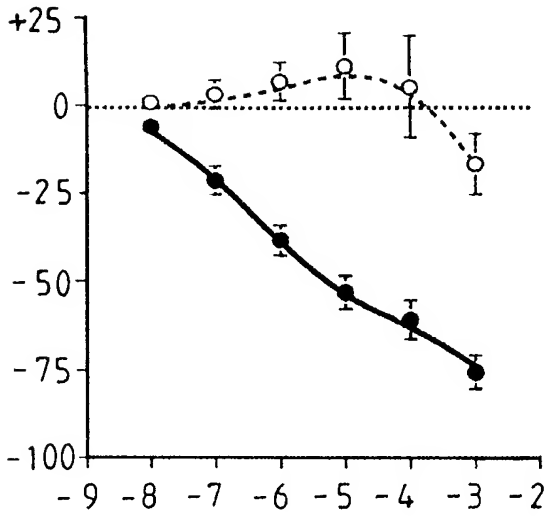

Fig. 5a

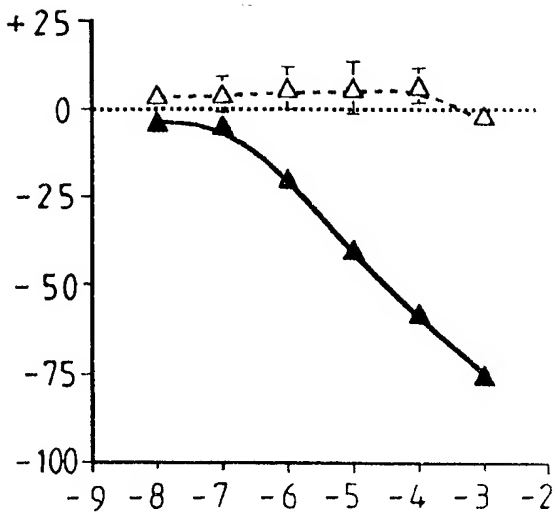

Fig. 5c

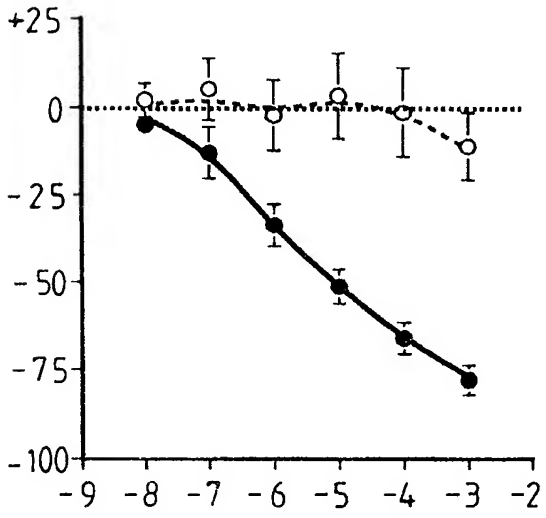

Fig. 5b

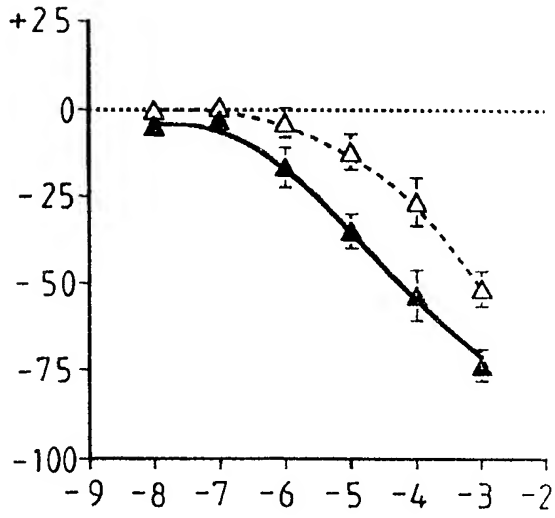

Fig. 5d

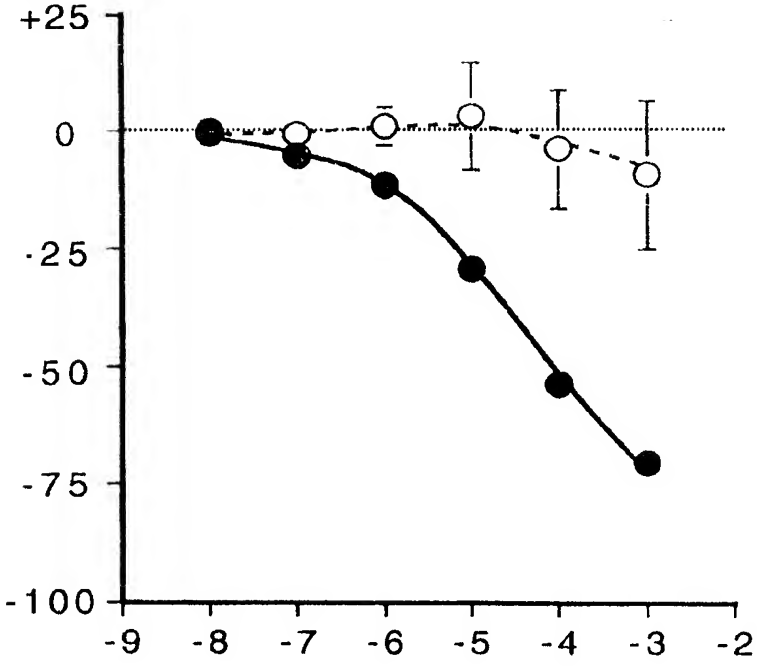

Fig. 6a

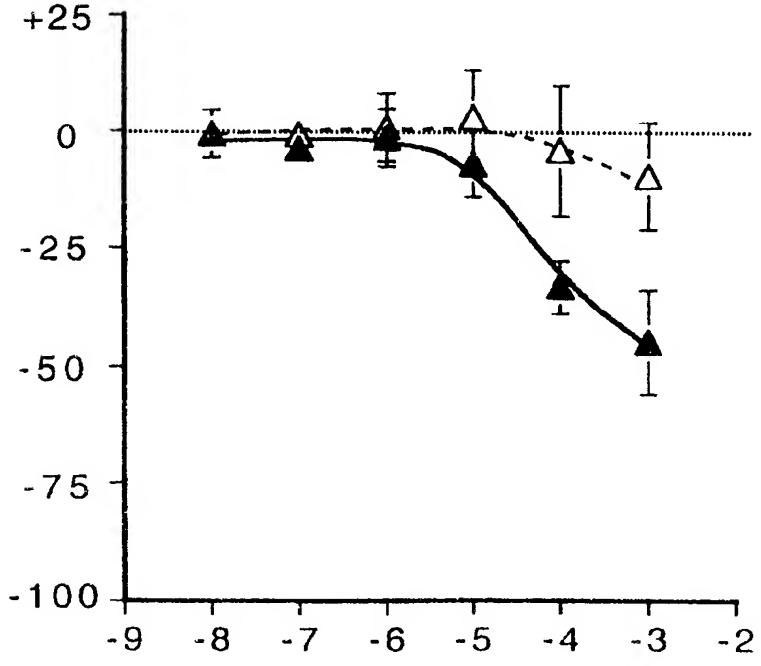

Fig. 6b

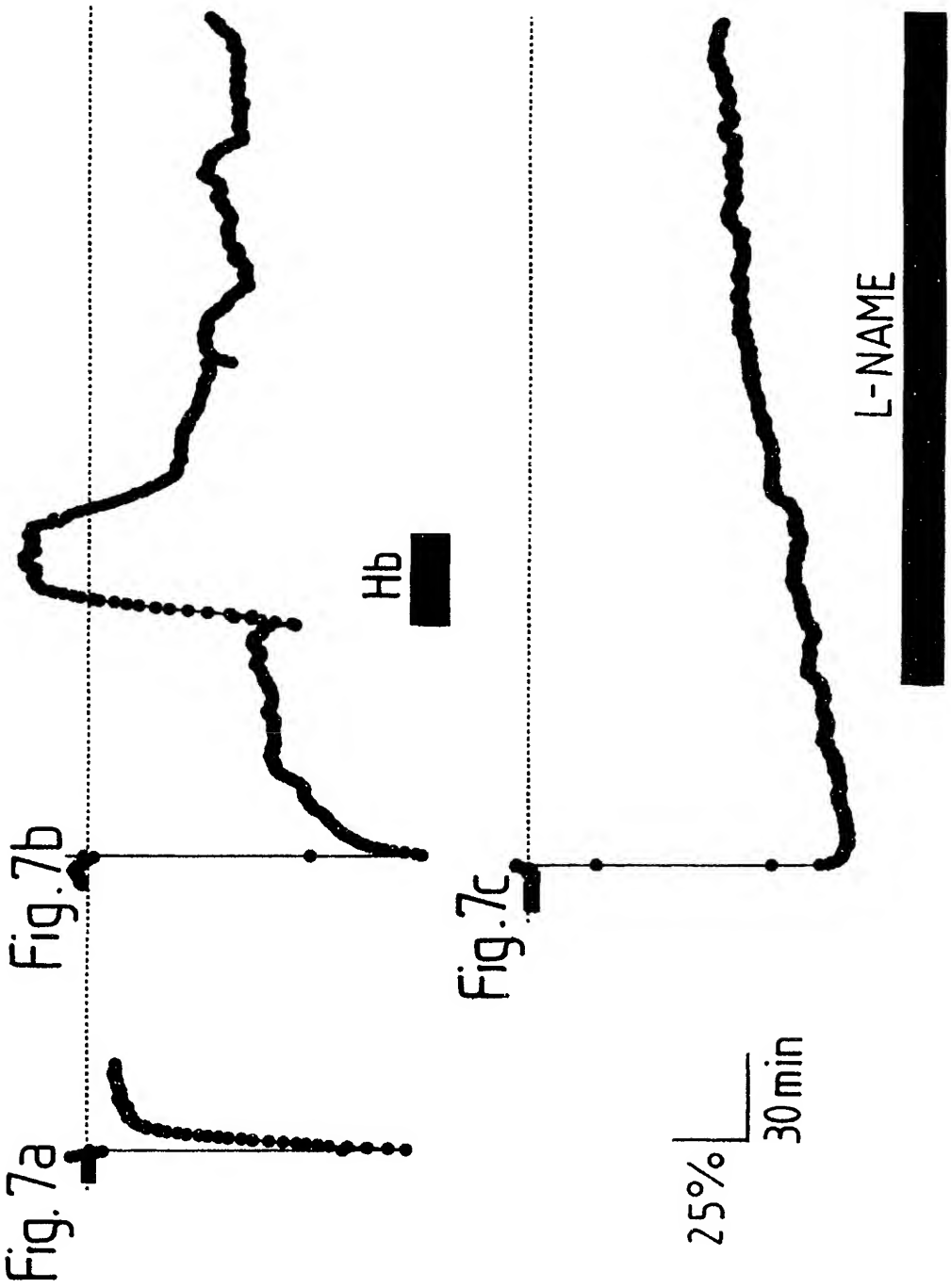

Fig. 7a

Fig. 7b

Fig. 7c

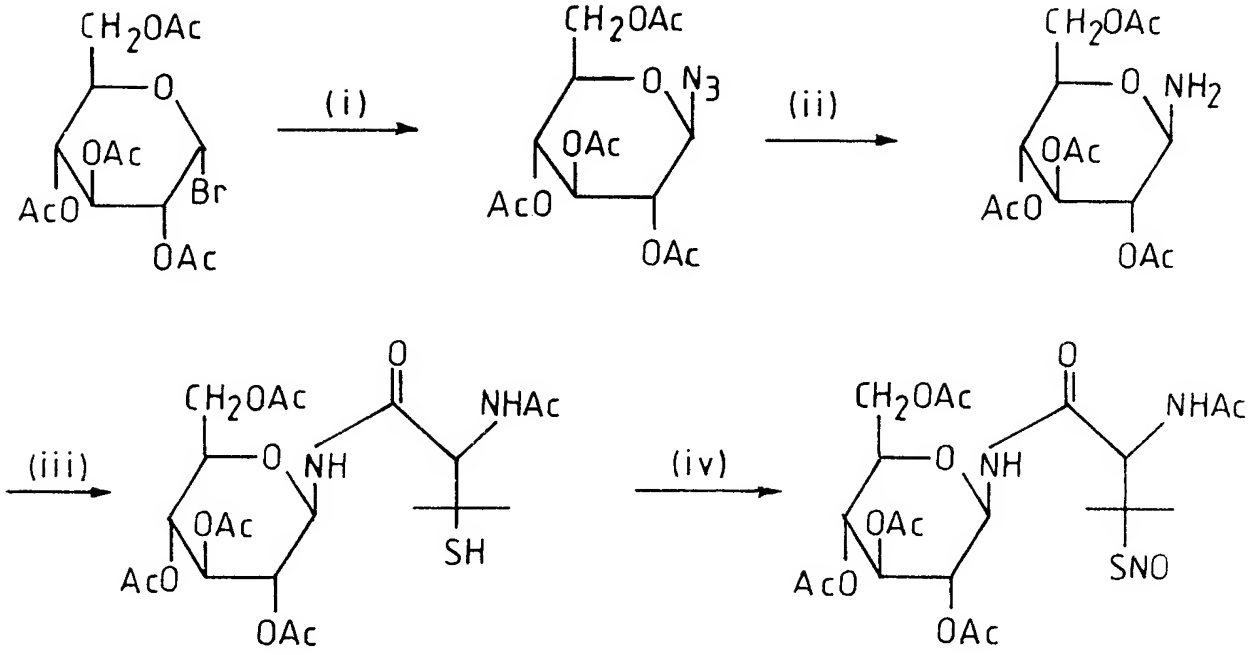
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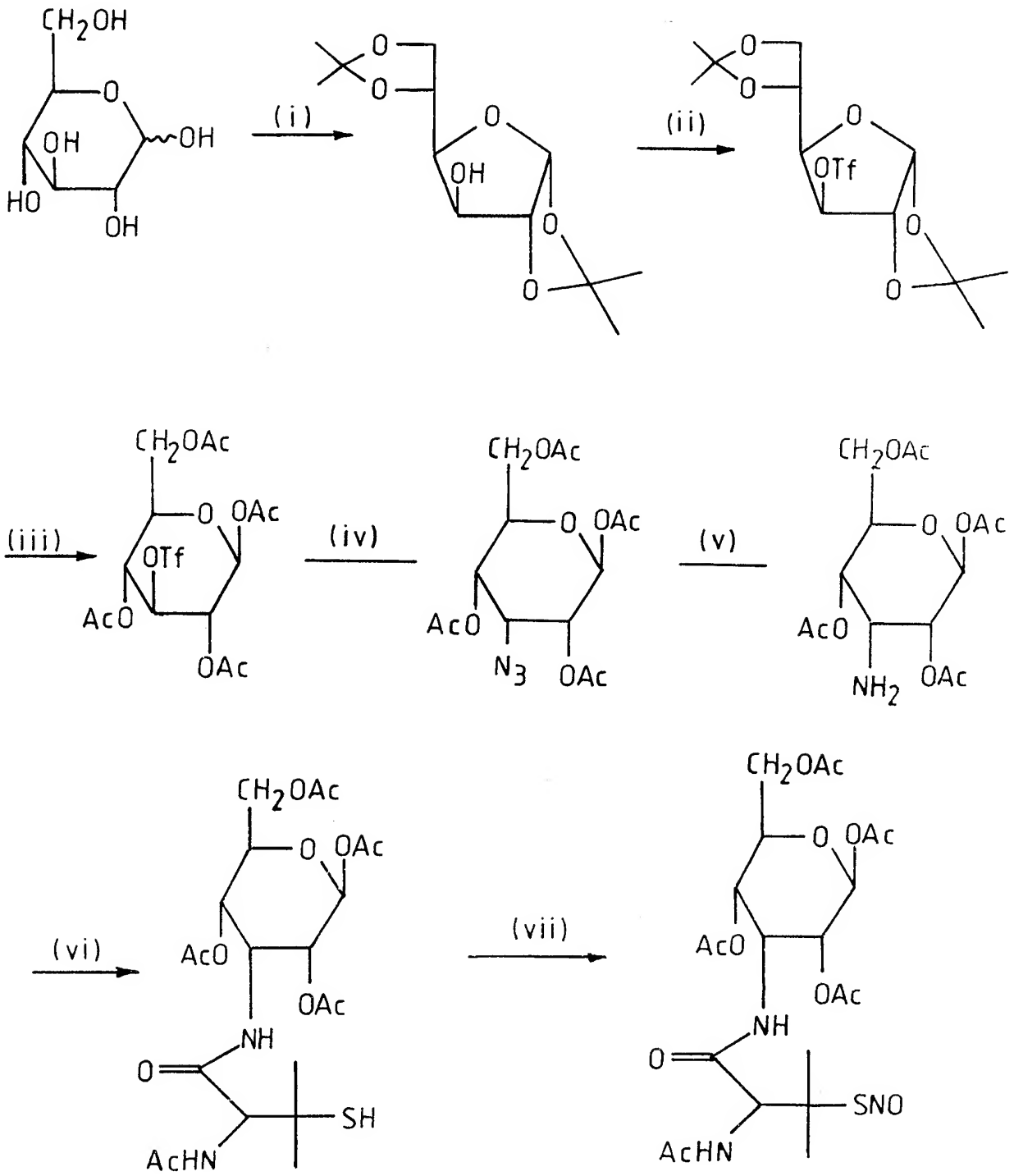
Fig. 8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AcHN

Fig. 9

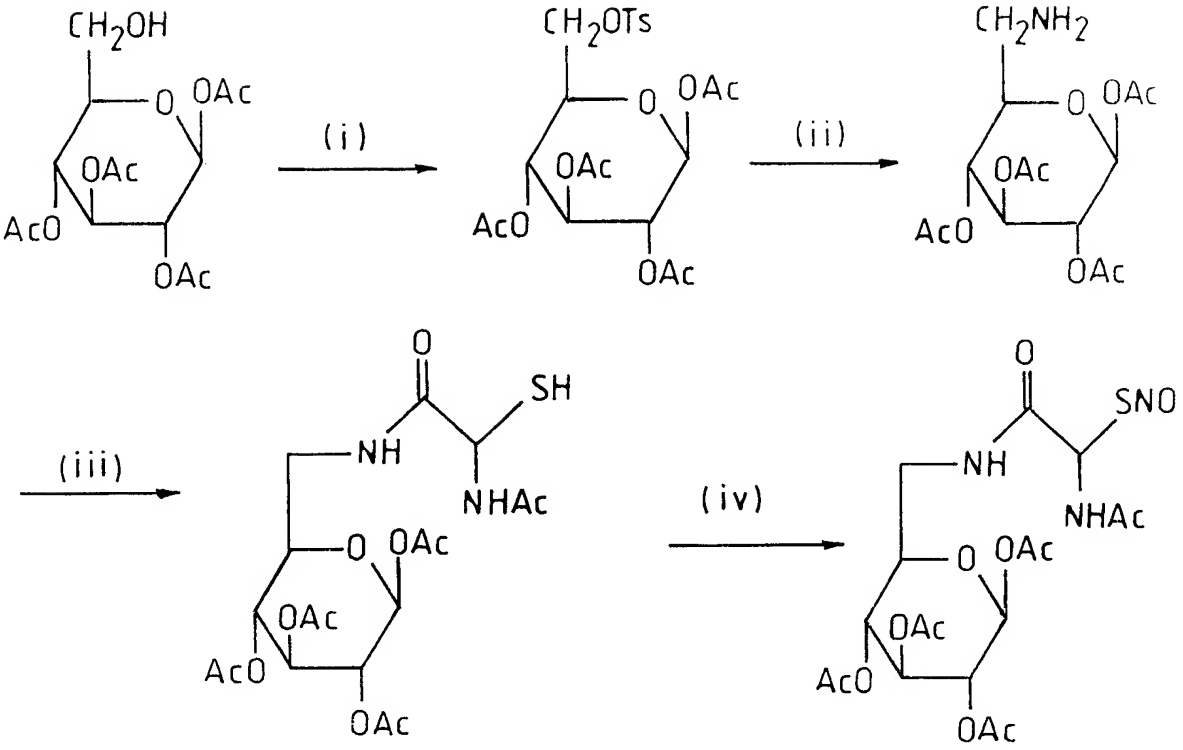
CH2OH
OAc
OAc
AcO
OAc
(i)
CH2OTs
OAc
OAc
AcO
OAc
(ii)
CH2NH2
OAc
OAc
AcO
OAc
(iii)
O
SH
NH
NHAc
OAc
OAc
AcO
OAc
(iv)
O
SNO
NH
NHAc
OAc
OAc
AcO
OAc

Fig. 10

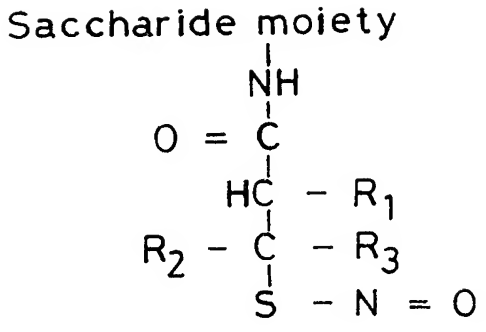

Fig. 11

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 97/03034

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H3/04 C07H3/06 A61K31/70

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J.RAMIREZ ET AL.: "Glyco-S-Nitrosothiols, a Novel Class of NO Donor Compounds." BIOORANIC AND MEDICINAL CHEMISTRY LETTERS, vol. 6, no. 21, 1996, pages 2575-2580, XP002056555 see the whole document --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1998	- 4. 03. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scott, J

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/GB 97/03034

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 125, no. 5, 29 July 1996 Columbus, Ohio, US; abstract no. 48272s, R.A.BUTLER ET AL.: "The Transdermal Delivery of an NO donor Drug : A New Approach to Raynaud's Syndrome." page 25; column 1; XP002056556 see abstract & PORTLAND PRESS PROC. (BIOLOGY OF NITRIC OXIDE PART 5), vol. 10, 1996, page 184 ---	1
A	GASTON B ET AL: "ENDOGENOUS NITROGEN OXIDES AND BRONCHODILATOR S-NITROSOTHIOLS IN HUMAN AIRWAYS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 23, December 1993, pages 10957-10961, XP000608469 see abstract ---	1
A	GIBSON A ET AL: "AN INVESTIGATION OF SOME S-NITROSOTHIOLS, AND OF HYDROXY-ARGININE, ON THE MOUSE ANOCOCCYGEUS" BRITISH JOURNAL OF PHARMACOLOGY, vol. 107, no. 3, 1992, pages 715-721, XP000615626 see abstract ---	1
A	IGNARRO L J ET AL: "MECHANISM OF VASCULAR SMOOTH MUSCLE RELAXATION BY ORGANIC NITRATES, NITRITES, NITROPRUSSIDE AND NITRIC OXIDE: EVIDENCE FOR THE INVOLVEMENT OF S-NITROSOTHIOLS AS ACTIVE INTERMEDIATES" JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 218, no. 3, September 1981, pages 739-749, XP000646163 see abstract ---	1

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INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/GB 97/03034

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 15-18 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/GB 97/03034

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	IGNARRO L J ET AL: "MECHANISM OF VASCULAR SMOOTH MUSCLE RELAXATION BY ORGANIC NITRATES, NITRITES, NITROPRUSSIDE AND NITRIC OXIDE: EVIDENCE FOR THE INVOLVEMENT OF S-NITROSOTHIOLS AS ACTIVE INTERMEDIATES" JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 218, no. 3, July 1981, pages 739-749, XP000646500 see abstract ---	1
A	SMITH M P ET AL: "IN VITRO VASORELAXANT AND IN VIVO CARDIOVASCULAR EFFECTS OF S-NITROSOTHIOLS: COMPARISON TO AND CROSS TOLERANCE WITH STANDARD NITROVASODILATORS" METHODS AND FINDINGS IN EXPERIMENTAL AND CLINICAL PHARMACOLOGY, vol. 16, no. 5, 1 June 1994, pages 323-335, XP000563847 see abstract ---	1
A	ARNELLE D R ET AL: "NO+, NO., AND NO- DONATION BY S-NITROSOTHIOLS: IMPLICATIONS FOR REGULATION OF PHYSIOLOGICAL FUNCTIONS BY S-NITROSYLATION AND ACCELERATION OF DISULFIDE FORMATION" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 318, no. 2, 20 April 1995, pages 279-285, XP000607903 see abstract -----	1

1